

Drug and Multidrug Resistance among *Mycobacterium leprae* Isolates from Brazilian Relapsed Leprosy Patients

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Skin biopsy samples from 145 relapse leprosy cases and from five different regions in Brazil were submitted for sequence analysis of part of the genes associated with *Mycobacterium leprae* drug resistance. Single nucleotide polymorphisms (SNPs) in these genes were observed in *M. leprae* from 4 out of 92 cases with positive amplification (4.3%) and included a case with a mutation in *rpoB* only, another sample with SNPs in both *folP1* and *rpoB*, and two cases showing mutations in *folP1*, *rpoB*, and *gyrA*, suggesting the existence of multidrug resistance (MDR). The nature of the mutations was as reported in earlier studies, being CCC to CGC in codon 55 in *folP1* (Pro to Arg), while in the case of *rpoB*, all mutations occurred at codon 531, with two being a transition of TCG to ATG (Ser to Met), one TCG to TTC (Ser to Phe), and one TCG to TTG (Ser to Leu). The two cases with mutations in *gyrA* changed from GCA to GTA (Ala to Val) in codon 91. The median time from cure to relapse diagnosis was 9.45 years but was significantly shorter in patients with mutations (3.26 years; $P = 0.0038$). More than 70% of the relapses were multibacillary, including three of the mutation-carrying cases; one MDR relapse patient was paucibacillary.

There is no doubt about the efficiency of the currently used multidrug therapy (MDT) scheme for treatment of leprosy, as demonstrated by the strong decrease in disease prevalence since its implementation and the low number of reported relapse cases (18). However, there has been a scarcity of in-depth studies of relapse occurrences in recent decades (27). As is known, differentiating diagnosis of relapse and reactional states poses some difficulties in the field, being responsible for under- or overdiagnosis of both disease stages. This is important because undiagnosed relapse cases could contribute to continuing disease transmission. In addition, hardly any data on the contribution of emergence of drug-resistant strains of *Mycobacterium leprae* to leprosy relapses exist.

Diaminodiphenylsulfone (DDS), also called dapsone, was the first drug to be effective against leprosy worldwide, and the first cases of resistance to dapsone were detected in 1964 and involved two single nucleotide polymorphisms (SNPs) in the gene *folP1*, located in codons 53 and 55 (8, 9, 14, 29). Rifampin is the key component of the standard multidrug regimen used for treatment of leprosy, and it has been shown that PCR-based DNA sequence analysis of the *rpoB* gene of *M. leprae* was in full concordance with rifampin susceptibility testing in the mouse footpad system (17, 30). In addition to dapsone and rifampin, ofloxacin is also used for leprosy treatment and is a quinolone with an action mechanism based on interaction with DNA gyrase (2); SNPs in *gyrA* and *gyrB* confer resistance or hypersensitivity to quinolones (15). Although there is not yet an official definition of multidrug resistance (MDR) in leprosy, in parallel with tuberculosis, we adopt this terminology when we encounter resistance to rifampin and one other drug of the standard MDT regimen.

Emerging drug resistance has been observed among many diseases caused by bacteria, and this could pose a challenge for the

treatment of leprosy, a neglected disease with a minimal therapeutic arsenal (22). Brazilian studies show relapse rates below 1% (12, 26), and drug resistance does not seem to be an important problem in the country (10, 21). Nonetheless, a pilot project for optimal detection of relapse and the contribution of drug resistance among leprosy patients of five states in Brazil was started in 2006 (26), in parallel with the initiative of the World Health Organization (WHO) to perform global surveillance of drug resistance in leprosy in 2008 (36).

MATERIALS AND METHODS

Study design and patients. A prospective study for detection of relapse in leprosy patients was designed for more accurate determination of the frequency of relapse by drug resistance among Brazilian leprosy patients, based on evaluation of DNA sequencing in samples from 145 leprosy patients, collected during 2006 to 2008, in five states to which leprosy is highly endemic, including Rio de Janeiro, Espírito Santo, Amazonas, Pará, and Ceará (26). All patients were examined by experienced dermatologists in six state reference units in order to guarantee the quality and uniformity of these procedures. Leprosy relapse detection was based on standardized and optimized diagnostic procedures and criteria for definition of relapse (4) and with inclusion criteria being suffering from active clinical lesions of leprosy, as confirmed by smears and histopathological exams, being considered cured from the first disease course after having undergone the

Received 30 November 2011 Returned for modification 29 December 2011
Accepted 29 March 2012

Published ahead of print 11 April 2012

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doi:10.1128/JCM.06561-11

Brazilian Leprosy Program treatment regimens. Regarding the official treatment regimens from the National Leprosy Program, it is necessary to clarify that Brazil, before adopting the WHO MDT treatment schemes in 1986 (24 doses), used a scheme called DNDS that consisted of 90 daily doses of 600 mg of rifampin, followed by daily doses of 100-mg dapsone monotherapy, up to 5 years and until slit skin smears became acid-fast bacillus (AFB) negative. For each relapse case, a control case, being a new leprosy case of the same sex, clinical form, and municipality of residence and belonging to the same treatment cohort, was selected from the National Information System for Notification of Diseases (SINAN) and enrolled for clinical and laboratory examinations.

This study was approved by the Ethical Committee of Research of the Federal University of Rio de Janeiro (HUCFF/UFRJ) (no. 019/06). Written consent was obtained from individual subjects by having them sign a standard Brazilian form before being admitted in the study. The epidemiologic, clinical, and demographic data collected from each participant center were stored and analyzed at the UFRJ, using the software program Strata 9.0.

Slit skin smear and histopathology of skin biopsy specimens. As part of the diagnostic procedure, slit skin smear samples were collected from four different body sites at the time of diagnosis of disease relapse, and a skin biopsy was done according to standard recommendations (4). After being cut in half, one part of the skin biopsy specimen was prepared for histopathology exam, and the other half was immersed in 70% ethanol for genetic analysis. In order to standardize the histopathology procedure and reporting of results, a consensus meeting was held with the histopathologists from the participating reference centers and a standard protocol was elaborated.

Extraction of nucleic acids. For extraction of nucleic acids, the ethanol was removed from the biopsy specimen, and the latter was rehydrated, cut into small pieces, and subjected to DNA extraction and purification using the Qiagen DNeasy Blood & Tissue kit (Invitrogen do Brasil). In brief, 180 μ l of ATL buffer and 20 μ l of proteinase K from the kit were added to the biopsy specimen and subjected to vortex mixing, and after overnight incubation at 56°C, DNA was purified using a spin column from the kit as described by the manufacturer.

Amplification and sequencing analysis of part of *rpoB*, *folP1*, *gyrB*, and *gyrA*. Part of the genes *rpoB*, *folP1*, and *gyrA* was analyzed by direct sequencing of PCR products generated using conditions described previously, using the amplification primers *MrpoBF* and *MrpoBR* (31), *folP1F* and *folP1R* (38), and *gyrANF* and *gyrANR* (5, 11, 23) and using touch-down amplification conditions described previously (11). Each PCR mixture contained at least one negative control, and after verification of PCR product quantity and quality on a 3% agarose gel, amplicons were purified using the ChargeSwitch PCR clean-up kit (Invitrogen do Brasil) and sequenced using the same primers as those for generating the PCR fragment of each gene, using the ABI BigDye 3.1 Terminator ready reaction kit (Applied Biosystems do Brasil). For characterization of the *gyrA* SNP at position 297, we followed the approaches described previously (11). Sequences were generated on an ABI 3730 genetic analyzer (Applied Biosystems) and compared with the *M. leprae* sequences NC002677 and z14314 (*rpoB*), AL023093 (*folP1*), and NC002677 (*gyrA*), available at GenBank (<http://www.ncbi.nlm.nih.gov/sites/entrez/>), and for SNP analysis, sequences were introduced into SeqScape. Control DNAs were purified from *M. leprae* NHDP-63 (kindly donated by Patrick Brennan, Colorado State University), and the plasmids *folP101*, -102, and -103 (a gift from Dianna Williams, Louisiana State University). Following the recommendations of the WHO Global Surveillance of Drug Resistance in Leprosy Protocol, samples with mutations suggestive for drug resistance as determined at FIOCRUZ were sent for blind sequence evaluation to M. Matsuoaka at the Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan.

In order to verify the presence of inhibitors in the processed biopsy samples, 23 biopsy samples that gave no PCR product in the *gyrA* system were subjected to a reconstitution experiment to verify the presence of

TABLE 1 Characteristics of relapse patients^a

Variable	Value for patients
Sex [no. (%) of patients]	
Male	105 (72.4)
Female	40 (27.6)
Clinical form [no. (%) of patients]	
MB	102 (70.3)
PB	43 ^b (29.7)
Treatment regimen of first disease course [no. (%) of patients]	
MDT MB 12 ^b	22 (15.1)
MDT MB 24	57 (29.3)
MDT PB	31 (21.3)
ROM ^c	2 (1.3)
DNDS + MDT 24	7 (4.8)
DNDS	14 (9.6)
Substitutive regimen ^d	12 (8.2)
Close contact with leprosy case [no. of patients/total (%)]	38/120 (31.6)
Age [median value, yrs (SD)]	47.5 (10.5)
Time from cure to relapse [median value, yrs (SD)]	
All cases	9.45 (4.95)
DR-MDR cases	3.26 (2.62)
Bacillary index [median value (SD)]	2.85 (1.87)

^a *n* = 145 relapse cases.

^b Multidrug therapy with the number of doses between 12 and 24.

^c ROM, rifampin plus ofloxacin plus minocycline.

^d Replacement of rifampin by ofloxacin or of dapsone by clofazimine or combined use of rifampin and clofazimine without dapsone. Statistical analysis was performed using Fisher's exact test.

eventual PCR inhibitors. For this, these samples were submitted to the PCR using the same conditions as described above, except for the addition of 1.5 ng of NHDP-63 DNA to each PCR mixture. For evaluation of inhibition, the PCR signal for reactions with biopsy sample was compared to that for reconstituted samples without biopsy sample and two positive controls (without reconstitution), as for the earlier PCR experiments. We used three interpretation criteria, with results having either (i) similar or (ii) less signal than the control samples or (iii) no amplification at all.

RESULTS

General patient data. Clinical data confirmed that 145 patients suffered from leprosy relapse, and their characteristics are summarized in Table 1. All of these patients presented the inclusion criteria, having been considered cured after completing the official treatment regimen (Brazil/DNDS or WHO/MDT) and having developed a second course of active leprosy disease, as confirmed by bacilloscopic and histopathological examination, also allowing the classification of the clinical form. Most cases (70%) were multibacillary (MB), while the rest were paucibacillary (PB); among the latter, the majority (88%) were borderline tuberculoid. The bacilloscopy index (BI) of the MB cases ranged between 0.25 and 6.0, with an average of 2.85. The average incubation period from cure to relapse diagnosis was 9.45 years, ranging between 1.5 and 25 years, and was significantly shorter in the four resistant cases (3.26 years; *P* = 0.0038), ranging between 1 month and 6.6 years. In addition, two of these cases had been subjected to more than one treatment regimen. Gender analysis showed that males

TABLE 2 Results of DNA sequencing and mutations in the *folP1*, *rpoB*, and *gyrA* genes of *M. leprae*^a

Gene	No. (%) of samples with conclusive sequencing for IBCAT of ^b :			P value ^c	No. (%) of samples with ^d :	
	0	1	2		SNP present	No SNP
<i>folP1</i>	4/60 (6.6)	22/41 (53.7)	31/44 (70.5)	<0.001	3 (5.3)	54 (94.7)
<i>rpoB</i>	5/60 (8.2)	19/41 (46.3)	33/44 (75.0)	<0.001	4 (7)	53 (93)
<i>gyrA</i>	18/60 (29.5)	27/41 (65.9)	32/44 (72.7)	<0.001	2 (2.6)	75 (97.4)

^a Total no. of relapsed cases: 145.^b IBCAT, categorized bacillary index (0, IB = 0; 1, IB > 0 and < 3+; 2, IB > 3+).^c Each P value is for the three groups as calculated with Fisher's exact test.^d SNPs are drug resistance related only.

(72.4%) were more affected than females (26.4%), and the median age of all cases at time of diagnosis of relapse was 47.5, ranging from 13 to 96 years (Table 1).

Upon analyzing treatment regimens, we observed that most of the MB first-disease cases had been treated with the MDT/WHO scheme, having completed either 24 or 12 doses, as adopted by the National Program; some MB cases, however, instead of having received 12 doses, had been subjected to one of the following: (i) a number of doses that varied between 12 and 24, as a consequence of the reduction of MDT treatment from 24 to 12 doses, as recommended by WHO, (ii) the DNDS regimen only, or (iii) the DNDR regimen and 24 doses of the MDT scheme. The last situation occurred in a considerable number of cases (Table 1) and also in three of the cases with drug-associated mutation (see Table 3).

Amplification and sequencing of *rpoB*, *folP1*, and *gyrA*. The results of amplification and DNA sequencing of part of the genes for *folP1*, *rpoB*, and *gyrA* are presented in Table 2. A total of 92 samples (63.4%) yielded sequence results for at least one gene fragment, and informative sequences were obtained for 57 cases (61.9%) for *folP1*, 57 cases (61.9%) for *rpoB*, and 77 cases (83.6%) for *gyrA*. Drug-associated SNPs were detected among 3 of the 57 samples for *folP1* (5.3%), 4 of the 57 samples for *rpoB* (7%), and 2 of the 77 samples for *gyrA* (2.6%). In addition, a statistically significant difference was observed between BI and sequence results (Table 2).

Among the 23 biopsy samples that were tested for the presence of PCR inhibitors, 21 had positive BI and 1 sample had a BI of 0, and for another sample we had no information on the BI. Among these samples, eight (35%) showed a PCR signal similar to that of

the positive controls, nine (39%) give weaker signals, and six (26%) gave no PCR product at all (data not shown), meaning that 65% of this sample selection showed some level of PCR inhibition for *gyrA* (data not shown). We did not test PCR inhibition in the PCR systems for *rpoB* and *folP1*.

Regarding the nature of the SNPs, the three changes in *folP1* were always a transition from CCC to CGC in codon 55 (Pro to Arg); in the case of *rpoB*, all occurred at codon 531, with two presenting a change from TCG to ATG (Ser to Met), one from TCG to TTC (Ser to Phe), and one from TCG to TTG (Ser to Leu); the two cases with mutations in *gyrA* presented a transition from GCA to GTA (Ala to Val) in codon 91. On the patient level, mutations suggestive of drug-resistant strains were observed in four cases, including one patient with a mutation in *rpoB* only, suggesting monoresistance to rifampin, one case with SNPs in both *folP1* and *rpoB*, suggestive of multiple drug resistance (MDR) for rifampin and dapson, and two cases with mutations in *folP1*, *rpoB*, and *gyrA*, strongly suggestive of MDR against the three main antileprosy drugs (Table 2). The sequence results obtained with the four cases that presented SNPs at Fiocruz were confirmed by M. Matsuoaka in the Leprosy Research Center, National Institute of Infectious Diseases, Japan.

In addition to the drug resistance-associated SNP in *gyrA*, this gene fragment also presented a synonymous SNP in position 297, and as demonstrated in Table 2, among the 77 samples that were sequenced, 57 (74.03%) presented the C allele, while 20 samples (25.97%) had the T allele. The four cases with drug resistance-associated SNPs presented the C allele.

Characteristics of patients with mutated strains. Table 3 summarizes the data from DNA sequencing and mutations found in the four patients, three being MDR. The first three cases were residents in former colonies for leprosy patients in the Amazon region, and all were subjected to the aforementioned DNDS regimen in their first disease episode. Case one, from the state of Para, presented the most characteristic resistance features, since his treatment failed in a second treatment course (first the DNDS/Brazil regimen and then two courses of MDT/WHO). His last treatment course ended in 2007, while he presented active lepromatous leprosy (LL) disease in the beginning of 2008. The two cases from Amazonas had also undergone two complete treatment schemes before diagnosis of relapse, and their clinical features provoked the suspicion of drug resistance (DR). For the first of these two patients, a mutation on *gyrA* was found, and we discovered that at the end of the

TABLE 3 Summary of drug-resistant relapse cases^a

Case no. (state ^b)	Sex ^c	Age (yrs)	CF	BI	His	Treatment	IP	Result of DNA sequencing ^d		
								<i>folP1</i>	<i>rpoB</i>	<i>gyrA</i>
1 (PA)	M	49	LL	5	LL	DNDS-MDT 24	1 mo	55, CCC → CGC (Pro → Arg)	531, TCG → ATG (Ser → Met)	No mutation
2 (AM)	M	63	LL	4,5	LL	DNDS-MDT 24	3.2 yrs	55, CCC → CGC (Pro → Arg)	531, TCG → TTC (Ser → Phe)	91, GCA → GTA (Ala → Val)
3 (AM)	M	46	BL	3,5	LL	MDT 24	3.3 yrs	No mutation	531, TCG → TTG (Ser → Leu)	No mutation
4 (ES)	M	38	BT	6,6	BT	MDT 12	6.6 yrs	55, CCC → CGC (Pro → Arg)	531, TCG → TTC (Ser → Phe)	91, GCA → GTA (Ala → Val)

^a CF, clinical form; BI, bacilloscopic index; His, histopathologic diagnosis; IP, incubation period of relapse; Pro, proline; Arg, arginine; Ser, serine; Phe, phenylalanine; Leu, leucine; Met, methionine; Ala, alanine; Val, valine; MDT/MB 24 (WHO), rifampin (RMP) (600 mg/month) + clofazimine (CLZ) (300 mg/month) (supervised) + DDS (100 mg) + CLZ (50 mg/day), during a period between 12 and 18 months; DNDS (Brazil), rifampin (RMP) (600 mg/day, 90 days) + dapson (DDS) (100 mg/day up to 5 years until AFB negative). Case one received three treatment courses.

^b PA, Pará; AM, Amazonas; ES, Espírito Santo.^c M, male.^d Codon number, mutation.

second scheme (MDT/WHO), this patient also had received ofloxacin but not according to a standard treatment scheme. Finally, the fourth and quite intriguing case from the state of Espírito Santo, southeast Brazil, was diagnosed with borderline lepromatous leprosy (BL) during first disease, presenting positive BI, but was negative in the second disease course, 7 years later, and classified as suffering from the borderline tuberculoid leprosy (BT) form. Although this patient presented a resistance-associated SNP in *gyrA*, we found no history of treatment with ofloxacin, and eventual reinfection by an ofloxacin-resistant strain acquired from his relatives could be possible. Among the 145 patients, 31% informed that they had relatives that were diagnosed for leprosy within 5 years before relapse diagnosis (Tables 1 and 3).

DISCUSSION

The efficiency of the WHO MDT scheme for leprosy treatment is supported by the dramatic decreases in disease prevalence and the low relapse rates in short and medium time frames. Therefore, relapse has not been considered a problem, and organization of studies of this disease characteristic was somewhat neglected, leading to the recent WHO initiative to organize a resistance surveillance project for relapse cases, 26 years after having started MDT. This was possible due to the development and standardization of molecular genotyping procedures of genes associated with drug resistance (5, 16, 23, 24, 38, 39).

After the introduction of relapse surveillance by the WHO, many of the countries of endemicity reported leprosy relapses. In addition, evaluation of the contribution of drug resistance under an international network has been implemented, focusing on MB relapse cases (36). For good-quality data on relapse rates, in addition to laboratory technology, uniformity of clinical criteria relapse diagnosis is important and needs to be standardized within and among countries. Although it not so difficult to diagnose leprosy relapse during the late MB disease form, recognition of relapse is not so easy during early disease, especially in the borderline spectrum cases of disease and under field conditions (19, 20).

In the present study, 29.7% of the relapses were PB cases, 88.3% of these being BT, and this after clinical examination by experienced leprologists and histopathological confirmation by three different pathologists. This was also the case for the BT patient that presented mutations in the *M. leprae* genes *rpoB*, *folP*, and *gyrA*, and possibly this patient, although being MDR, presented this disease form because he was diagnosed very soon after developing relapse, had a better immune host defense response, or had a different strain causing relapse, either by reinfection or strain selection, as observed in a considerable number of relapse cases in another study (11). On the other hand, selection of a particular part of the *M. leprae* population that caused first disease as being responsible for relapse is in accordance with the work of Toman in 1981 (35) and Colston et al. in 1987 (7), raising the possibility that “persistent” *M. leprae* could cause relapse in a large proportion of patients, the persistent bacilli presenting a metabolic state that resists the drug without the presence of drug-associated mutations, also suggested by Pattyn (28) and Balagon et al. (1).

Suspicion of DR or MDR in leprosy is raised mainly because of maintenance of clinical symptoms, with or without evaluation of the presence of bacilli in skin smears and confirmation by growth in the footpads of mice fed with antibiotics. Bacteriological anal-

ysis by smear microscopy is not always reliable, however, and advances in the elucidation of molecular events responsible for drug resistance in mycobacteria have allowed the development of alternative tools for drug resistance screening (6). However, due to the need of technical expertise and specialized equipment, this technique is executed in a limited number of centers in Brazil (38). Nonetheless, SNP detection seems to be more sensitive and is certainly much quicker for detecting DR than the mouse footpad-based technique (23, 33). In two very recent studies, sequence analysis for DR in Latin American leprosy patients was reported, the first report presenting two cases with SNPs in *rpoB* and one case in *gyrA*, suggestive for resistance against rifampin and ofloxacin, respectively, among 38 Mexican cases, suggesting the possible reemergence of DR leprosy in a country where leprosy was considered eliminated (22). The second study included 230 mostly new leprosy cases, two being from Uruguay, 10 from Bolivia, 23 from Brazil, and 197 from Venezuela. Only two relapse cases presented SNPs in the three genes studied, one from Venezuela in *folP1* and one from Brazil in *folP1* and *rpoB* (34).

The mutations observed presently all have been reported in studies in other countries, including the changes in codon 531 of *rpoB*, causing an amino acid change from Ser to either Met ($n = 1$), Phe ($n = 2$), or Leu ($n = 1$), the SNP observed in *folP1* in codon 55 ($n = 3$), causing the change of Pro to Arg, and the mutation at codon 91 of *gyrA* ($n = 2$), leading to a change from Ala to Val. These SNPs had been described earlier in several reports, including those of Honoré and Cole (17), Williams et al. (37), Cambau et al. (6), and Gillis and Williams (14). In addition to the nonsynonymous SNP in *gyrA*, we observed the allele distribution in the relapse cases of a recently observed synonymous SNP at position 297 of *gyrA* (11, 25), showing that 74% of the cases carried *M. leprae* of the SNP type *gyrA* C at position 297. Our own previous data (11) and the recently published data from Singh et al. (34) showed the correlation of the synonymous SNP *gyrA* 297T type with the SNP type 3 and of SNP *gyrA* C with type 1 or 4 defined by Monot et al. (25). Previous data showed the higher frequency of the SNP3 type in southeast Brazil (13) and Latin America (34), and the prevalence of the SNP *gyrA* C could be due to sampling from other regions of Brazil.

We did not obtain PCR products and good-quality sequences from all samples, and this is due partly to the inclusion of samples with low or zero bacterial counts and to the presence of PCR inhibitors, as evidenced by the reconstitution experiment. Indeed the presence of PCR inhibitors in skin biopsy samples has been described before (32).

The significant difference between the period of time between first disease and relapse between resistant and nonresistant cases is in agreement with the work of Pattyn et al. (28), suggesting a difference in the incubation period in these two kinds of relapses. One MDR relapse case, however, showed such a short incubation period (1 month) that we suspect that this patient had not really been cured from his second disease course (Table 3). Our observation that all resistant cases were males is in agreement with findings of other studies (29, 30) and could be associated with the higher prevalence of males in MB leprosy and more frequent irregular self-administered drug intake (including quinolones) in males, causing mainly secondary resistance. This is supported by the recent observation of Singh et al. (34) showing the absence of primary drug resistance as demonstrated by the lack of drug-related mutations in strains from new leprosy patients. Indeed, three

out of four of the DR patients are from leprosy colonies that had received a previous Brazilian treatment regimen before MDT/WHO. Possibly, these cases, despite receiving regular monthly doses of the MDT/WHO scheme, might have been noncompliant regarding the daily self-administered dose of combined dapsone and clofazimine.

Although DR does not seem to be a problem in Brazil, one should note that the three older DR cases had skin lesions typical of leprosy and good access to a health unit and yet suffered from late diagnosis, strongly suggesting the need of inclusion of ex-colony areas as “loci” for epidemiological surveillance for relapse, as per norms defined by the Ministry of Health (4). Also, the observation of two cases of strains of *M. leprae* with MDR against the three most common drugs for treatment is concerning and could become a serious threat for leprosy control. In order to comply with the Global Surveillance of DR in Leprosy, the following had been recommended: (i) to provide a technical guideline from the National Hansen’s Disease (Leprosy) Control Programme (4) for establishment of relapse surveillance measures, (ii) to include the study of drug resistance, (iii) to provide recommendations for the management of suspected relapse cases, and (iv) to design a specific investigation form for the cases reported as relapse in the SINAN national information system (3). In addition, we suggest the implementation within the leprosy control program of monitoring of DR and MDR patients and their close contacts and organizing a reference framework.

Our data show that development of DR isolates of *M. leprae* is contributing to leprosy relapse in Brazil but that the following are alternative causes: (i) bacterial persistence, (ii) immunosuppression of the host, (iii) pregnancy, (iv) the presence of advanced leprosy, (v) reinfection, and (vi) factors associated with failures in operational health care, such as late diagnosis, inadequate or irregular treatment of the disease, and misclassification of earlier disease (11, 18, 19, 20). We admit, however, that a limitation of this study is the use of PCR sequencing for SNP detection, with limitations regarding the detection of eventual minor mutant populations. In addition, mutations outside the part of the genes that was sequenced could have been missed.

ACKNOWLEDGMENTS

This study was funded by CNPq/DECIT/MS-Brazil, grant N401296/2005-9.

The work was made possible by infrastructure of the Laboratory of Molecular Biology Applied to Mycobacteria and Leprosy Laboratory of FIOCRUZ, Rio de Janeiro, RJ, Brazil, UFRJ coordination, and the collaboration of many health professionals from all six outpatient reference health units and participants from five states of Brazil.

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